A novel family of bacterial sialic acid binding adhesins?

Undergraduate Research Thesis

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by

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ABSTRACT

Platelet binding is a critical step in the development of Infective Endocarditis (IE), an infection of the endocardium. However, the mechanisms utilized by IE causing species for binding to platelets remain understudied. Subacute IE, is associated with previously damaged heart valves and is often caused by the bacterial species *Streptococcus oralis. S. oralis* binds sialic acid, a host carbohydrate found on the surface of platelets. A novel sialic acid binding adhesin, AsaA, was identified in *S. oralis.* We identified orthologs of AsaA in two other bacterial species that cause IE, *Gemella haemolysans* and *Granulicatella elegans.* Our hypothesis is that AsaA mediates adherence of multiple species. *G. haemolysans* was selected for further study and shares 62% predicted amino acid identity with the non-repeat region of AsaA from *S. oralis. G. haemolysans* has rarely been studied, so we began by optimizing growth conditions. Binding of the species to platelets was consistently low which prevented the direct assessment of the role of *G. haemolysans* AsaA in adhesion. Given the same sialic acid binding specificities, the adherence of *S. oralis* to platelets can be competitively inhibited by recombinant proteins that bind terminal sialic acid. Hence, we recombinantly expressed the binding region of AsaA from *G. haemolysans* (AsaA_NRGh) and observed the impact of the protein on the adherence of *S. oralis* to platelets. AsaA_NRGh competitively inhibited adhesion of *S. oralis* to platelets in an AsaA dependent manner. This finding supports the hypothesis that *G. haemolysans* AsaA acts as an adhesin. If AsaA is a conserved mechanism of adhesion, a single treatment or preventative measure may target multiple IE causing species.

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CONTENTS

INTRODUCTION

Infective endocarditis (IE), an infection (usually bacterial) of the heart endocardium, continues to be associated with high rates of morbidity and mortality. The disease is associated with a 20 percent in-hospital mortality rate and 40 percent one year mortality rate post diagnosis (1-4). The incident rate of IE has not decreased over the past 20 years and may even indicate an upward trend (1-3,5). IE disproportionately impacts the elderly as the elderly populations more often have risk factors associated with the disease such as calcific valvular lesions and prosthetic valves (6). IE often requires hospital treatment, including intravenous antibiotics and surgery. An estimated 40 to 50 percent of patients infected with IE have to undergo surgery (3). Since there have been no significant improvements in the recent survival rate of patients diagnosed with IE, it is important we continue to study the mechanisms by which the disease develops. Understanding these mechanisms may point to a new preventative measure or treatment option.

There are two subsets of IE: acute and subacute. Acute IE is typically associated with previously normal heart valves and is rapid in presentation. Symptoms such as high fevers and sepsis are often associated with the rapid onset of the disease (7). Acute IE is commonly caused by staphylococcal species such as *Staphylococcus aureus.* (8). Alternatively, subacute IE is typically associated with previously damaged valves and is often caused by streptococcal species. Symptoms of subacute IE vary and include congestive heart failure and valvular incompetence. In comparison to acute IE, subacute IE is more gradual in presentation (3). In subacute IE, the damaged endocardial surface promotes binding of platelets and fibrin. Platelets and fibrin complex to form a nidus, a focus of infection, which then becomes infected with bacteria circulating in the blood. Bacteria may bind directly to this nidus or bind to circulating platelets which then adhere to the damaged endothelial surface. (9-11). Although it is unclear which path

bacteria follow, it is well accepted these bacteria bind to platelets. Electron microscopy confirmed bacterial adherence to platelets in a rabbit model for IE. (12). Once bacterial colonization has occurred growth of the bacteria in conjunction with host processes including activation of the coagulation system and deposition of fibronectin lead to vegetative growths. These vegetative growths result in the clinical effects of IE such as congestive heart failure (10,11,13). Previous studies have identified viridans group streptococci, which can enter the bloodstream from the oral cavity (3, 14-17), as common causes of subacute IE. It is widely appreciated that two viridans group streptococcal species *Streptococcus gordonii* and *Streptococcus sanguinis* bind to platelets. Adherence of these bacterial species to host carbohydrates on platelets is an important step in the development of IE. Specifically, these species bind to sialic acid, a host carbohydrate present on the surface of platelets (18-20). The binding of these species to sialic acid on platelets is mediated by serine-rich repeat proteins (SRRPs) (13, 18, 20-22). Mutagenesis of SRRPs found in streptococci have resulted in reduced pathogenesis of IE in a rat model (23-25). SRRPs are a family of bacterial adhesins that mediate adherence to host and bacterial surfaces (26-31). SRRPs contain two glycosylated serine-rich repeat (SRRs) regions that flank a non-repeat region (NRR) which is responsible for binding to host and/or bacterial surfaces. (27). Different SRRPs can bind to different receptors dependent on the domains within the NRR. SRRPs have been identified in both *S. gordonii* and *S. sanguinis* that contain a sialic acid-binding immunoglobin-type lectin (Siglec)-like domain within the NRR of the protein. In addition to sialic acid, SRRPs can bind to receptors including fibrinogen, DNA, and keratins. (27,28). Identifying the mechanisms by which bacteria bind to host carbohydrates on platelets may provide insight into future preventative measures or therapeutics.

The King Lab studies the mechanisms by which the viridans group streptococcal species *Streptococcus oralis,* causes subacute IE. *S. oralis* is a Gram-positive bacterium that is present in normal human oral microbiota. However, as a leading cause of subacute IE, it is also an important etiological agent of the disease (15,32). Previous studies performed by King lab with *S. oralis* indicate that similar to other virdians group streptococci, the species binds to host carbohydrates present on platelets. *S. oralis* is composed of three subspecies: *S. oralis* subsp. *oralis*, *S. oralis* subsp. *tigurinis* and *S. oralis* subsp. *dentisani* (33,34). All three subspecies have been associated with cases of IE (35-38). Investigation of the adherence mechanisms utilized by these subspecies will result in a better understanding of the disease.

Multiple isolates of *S. oralis* subsp. *oralis* encode Fap1, a serine-rich repeat protein (SRRP) that mediates binding of the bacterium to sialic acid. Similar to the SRRPs found in *S. gordonii* and *S. sanguinis,* Fap1 contains a Siglec-like domain which mediates binding of *S. oralis* subsp. *oralis* to platelets (30). Previous studies have demonstrated sialic acid as a conserved receptor across multiple isolates of *S. oralis.* However, some of these isolates capable of binding to sialic acid lack Fap1. This finding demonstrates that Fap1 is not essential for *S. oralis* binding to terminal sialic acid present on platelets. Isolates that lack Fap1 must bind via a distinct SRRP or in an SRRP-independent manner. To identify the adhesin responsible for binding to sialic acid, isolate IE12, one of the isolates that lacks Fap1, was genome sequenced. Genomic sequencing revealed that IE12 did not contain a SRRP. Comparative genomics and structural predictions were utilized to identify a cell wall anchored protein named AsaA (associated with sialic acid adhesion A) which contains two Siglec-like domains. To directly assess the role of AsaA in binding, an Δ*asaA* mutant was created. There was a significant reduction in the adhesion of the mutant which demonstrates AsaA is required for binding of IE12 to platelets. There is no further reduction in

adherence of the Δ*asaA* mutant with the enzymatic removal of sialic acid. This finding demonstrates AsaA is specifically required for binding to sialic acid on platelets. To further investigate the role of AsaA in adhesion, the NRR region of AsaA from isolate IE12 was recombinantly expressed (AsaA_NRSo). The ability of AsaA_NRSo to reduce IE12 adherence indicates AsaA is an adhesin. This claim is supported by the dose-dependent reduction in adherence of IE12 in the presence of increasing concentrations of AsaA_NRSo. These data demonstrate IE12 binds to sialic acid via novel adhesin, AsaA.

The identification of AsaA prompted the search for other IE causing bacterial species that encode for AsaA orthologs in order to determine if AsaA is a conserved mechanism of adhesion across different species. Orthologs of AsaA were identified in two other bacterial species that cause IE, *Gemella haemolysans* M341 and *Granulicatella elegans* ATCC 700633 (39,40).

G. elegans is a fastidious Gram-positive species and is a component of normal human oral microbiota. Prior to reclassification, the species was described as a nutritionally variant *Streptococcus* (41,42). Similar to *G. elegans*, *G. haemolysans* is a Gram-positive species and was also misidentified as a member of the streptococcal genus (43). This species is normally present in the upper respiratory, gastrointestinal, and genitourinary tract (39). Case reports have described *G. haemolysans* as a cause of subacute IE likely entering the bloodstream from the oral cavity (44-47).

The focus of this study was to examine the role of AsaA in binding to platelets across multiple bacterial species. We hypothesized that AsaA is a novel family of adhesins that mediates adherence of multiple species to sialic acid. Studies were focused on examining the role of AsaA in adhesion in *G. haemolysans.* If AsaA is a conserved mechanism of adhesion, a single treatment or preventative measure may target multiple IE causing species.

METHODS

Bacterial strains, culture, and media

Strains used in this study are listed in Table 1. *S. oralis* subsp. *oralis* and *G. haemolysans* were grown overnight at 37°C and 5% CO_2 on tryptic soy agar plates supplemented with 5% sheep's blood (Becton, Dickinson and Co., Sparks, MD). Broth cultures of *S. oralis* subsp. *oralis* were grown in Todd-Hewitt broth (Becton, Dickinson and Co.) supplemented with 0.2% wt/vol yeast extract (Becton, Dickinson and Co.) (THY). Broth cultures of *G. haemolysans* were grown in Brain Heart Infusion (BHI) supplemented with 5% fetal bovine serum (FBS) (Dworkin).

Escherichia coli strains were grown at 37°C in Luria-Bertani (LB) broth or LB agar plates. *E. coli* grown in broth was shaken at 200 rpm. As appropriate, the medium was supplemented with ampicillin (100 μg/ml) or kanamycin (500 μg/ml) (Thermo Fischer Scientific).

Unless otherwise specified, all chemicals, substrates, and enzymes were purchased from Sigma-Aldrich (St. Louis, MO).

Mutant Generation

Attempts were made to generate an insertion-deletion mutant of *G. haemolysans* via allelic exchange. First, a 1.5 kb fragment from the NRR of *asaA* from *G. haemolysans* M341 was amplified with primers A1 and A2. A Janus cassette was also amplified using primers B1 and B2. The *asaA* fragment was blunt-end ligated into pJet1.2/Blunt PCR cloning vector (Thermo Fisher Scientific) and the resulting plasmid was linearized with HincII. The Janus cassette was then blunt-end ligated with the linearized plasmid to generate plasmid pJETΔ*asaA*. *E. coli* Stellar competent cells (Clontech) were transformed with the ligation product and transformants were then selected for on LB agar plates supplemented with kanamycin. The construct was confirmed

via PCR. Attempts were made to transform *G. haemolysans* by growing bacteria in C+Y media to an OD_{600} (optical density measured at a wavelength of 600 nm) of 0.12 +/- 0.02. 50 μL of culture was added to 950 μL C+Y, 10 μL $CaCl_2$ (100 mM), and 100 ng of purified plasmid. The mix was incubated for 2 hours in a 37°C water bath and plated on TS agar plates with kanamycin. No transformants were observed.

Adherence Assays and Platelet Prep

Assays to determine bacterial binding to human platelets were adapted from Sullam et al (48). Blood was collected from healthy donors and no identifying information was collected. The blood was collected in 1:5 (vol/vol) acid citrate dextrose solution (56 mM trisodium citrate dehydrate, 65 mM citric acid, 100 mM dextrose). Platelet-rich plasma (PRP) was collected by centrifugation for 10 mins at 200 x g. PRP was removed with a serological pipette and diluted 1:4 (vol/vol) in platelet wash buffer (9.3 mM trisodium citrate dehydrate, 5.3 mM citric acid, 17.3 mM dextrose, 145.5 mM NaCl, pH 6.5). Platelets were collected by centrifugation for 10 mins at 700 x g and washed twice with platelet wash buffer. Platelets were resuspended in platelet wash buffer and fixed with 1% paraformaldehyde for 10 mins at room temperature. Platelets were then washed with PBS two times before being resuspended at $1x10_7$/ml. For adherence assays, each well of a 96 well microtiter plate was coated with 100 μl of platelets and incubated at 37°C for 1 hour. Following incubation, unbound platelets were removed by three washes with PBS. The control wells were coated with 1% bovine serum albumin (BSA) in PBS. All wells were blocked with 3% BSA in PBS and incubated for 1 hour at 37°C prior to adherence assays.

Wells were washed two times with PBS prior to the addition of bacteria. Where stated, AsaA_NRSo, AsaA_NRGh, or Fap1_NR was mixed with bacterial cells at the specified concentration. AsaA_NRGh was recombinantly expressed and purified as described below. AsaA_NRSo and Fap1_NR were recombinantly expressed and purified by a postdoctoral scientist in the King Lab, Meztlli Gaytan (30).

Bacteria were grown to an OD_{600} of 0.5 +/- 0.05 and approximately 1-2 x 10^5 bacteria in PBS were allowed to adhere at 37°C for 60 mins. Nonadherent bacteria were removed by three washes with PBS and adherent bacteria were lifted with 0.25% trypsin-1mM EDTA. Lifted bacteria were enumerated by serial dilution.

Adherence assays were performed in triplicate on at least three independent occasions and bacterial adherence was calculated as a percentage of the inoculum. The data are presented +/- the standard deviation (SD) and statistical significance was determined using a two tailed Student's *t*-test. Data points with *P* values ≤0.05 were considered significant.

Expression and Purification of AsaA_NRGh

Prior to this study, the NRR of *asaA* encoding amino acids 50-739 from *G. haemolysans* was amplified using primers A3 and A4. The fragment was cloned into pGex-5X-3 via In-Fusion and the product transformed into *E. coli* stellar. The transformants were selected for on LB plates supplemented with ampicillin. PCR and sequencing were utilized to confirm the expression construct.

BL21 competent *E. coli* (New England Biolabs, Ipswich, MA) were transformed with the above construct. Transformants were selected for on LB agar plates supplemented with ampicillin. Cultures were inoculated in 5 mL of LB broth supplemented with ampicillin overnight at 37°C.

The overnight culture was added to 100 mL of LB and grown at 37°C under constant shaking. Once the culture reached an OD_{600} of 0.6-0.8, 1mM Isopropyl β-D-1-thiogalactopyranoside (IPTG) was added to induce expression. The induced culture was incubated at 30°C for 4 hours. Cells were harvested by centrifugation at 5000 rpm for 10 mins and resuspended in 1/10 the original volume of PBS. Cells were lysed using a French press at 20 kPa. Cell debris was collected by centrifugation for 30 mins at 14000 rpm. The supernatant was separated from the cellular debris. The soluble protein in the supernatant was purified via affinity chromatography using Glutathione Sepharose 4B GST-tagged protein purification resin (GE Healthcare Life Sciences, Marlborough, MA), followed by elution with 50 mM tris-HCl, 10 mM glutathione and dialysis against PBS, yielding concentrated protein. The purified protein was run on an SDS-page gel to confirm its size (101.6 kDa) and purity. To quantify the protein its absorbance was measured at 280 nm.

Table 1: Strains used in this study

Strain or plasmid	Characteristic(s)/genotype[a]	Source or reference
Strain		
S. oralis* subsp. *oralis		
SN51445 (IE12)	Endocarditis isolate from an 81 year old female	GNRCS[a]
IE12Δ*asaA*	Δ*asaA*::*kan/rpsL+rpsL* (K56T), Kan^r	This study
G. haemolysans		
M341	Isolated from expectorated sputum for 19-year-old male patient with cystic fibrosis	BEI resources[b]
Escherichia coli		
Stellar	Cloning Host	Takara
BL21 (DE3)	Expression Host	NEB
Plasmids		
pJet1.2/blunt	Cloning vector, Amp^r	Thermo Fisher Scientific
pJetΔ*asaA*	pJetΔ*asaA*::*kan*; Amp^r Kan^r	This study
pGex-5x-3	Expression vector; Amp^r	GE Biosciences
pGex-5x-3 AsaA NRR	pGex-5x-3 containing amino acids 50-739 from the NRR of AsaA from *G. haemolysans*	This study

[a] German National Reference Center for Streptococci

[b] Strain M341, HM-239 was obtained through BEI resources, NIAID, NIH as part of the Human Microbiome Project

[c] Amp^r, ampicillin resistant; Kan^r, kanamycin resistant

Table 2: Primers used in this study

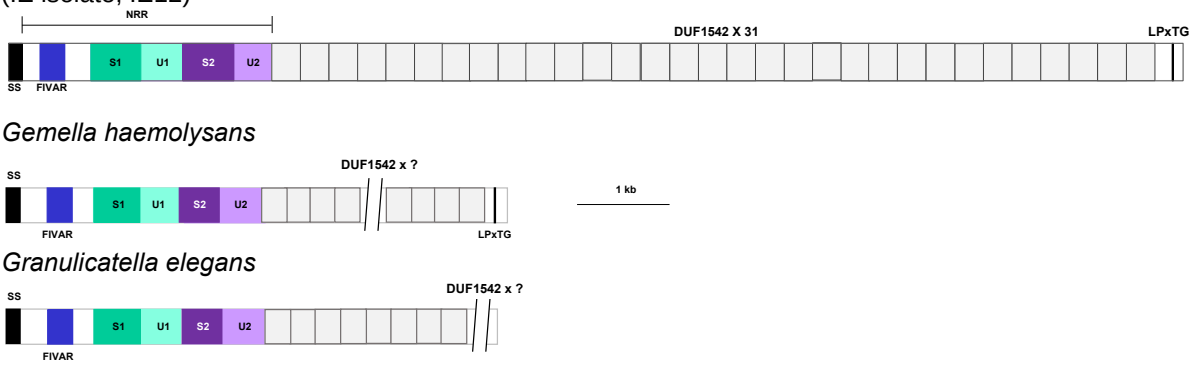
Target or Group	Name	Sequence 5' to 3'	Location (accession no.)
asaA	A1	AGGTTATGCGCTTTTCAATTCG	48689-48710 (GL883584.1)
	A2	GAAACAGAGCCTTCTCCACTCG	50653-50674 (GL883584.1)
	A3	GAAGGTCGTGGGATCAGTGAAGAAGTTTCAAAAAAC	49801-49821 (GL883584.1)
	A4	GATGCGGCCGCTCGATCATAAACTTACATATTCTGATA	51851-51888 (GL883584.1)
Janus	B1	GGGCCCCTTTCCTTATGCTT	2105–2086 (AF411920.1)
	B1	CCGTTTGATTTTTAATGGATAATG	773–796 (AF411920.1)

RESULTS

AsaA orthologs were identified in both *Gemella haemolysans* and *Granulicatella elegans*

S. oralis IE isolate IE12 can bind to sialic acid, but lacks SRRP Fap1. Sequencing of IE12 revealed it encodes for sialic acid adhesin, AsaA. Four of the seventeen sequenced strains identified as *S. oralis* subsp. *oralis* on the NCBI encode for AsaA. In IE12, directly upstream of *asaA* is *iga*_1 which encodes for a peptidase and directly downstream is *pabB* which encodes for an amino transferase. The discovery of this adhesin prompted the search for AsaA orthologs in other IE causing bacterial species to examine if AsaA is a conserved mechanism of adhesion. An NCBI BLASTp (protein-protein Basic Local Alignment Search Tool) with AsaA from *S. oralis* revealed AsaA orthologs in two IE causing species, *Gemella haemolysans* and *Granulicatella elegans* (49). IE12 AsaA as well as both AsaA orthologs contain a secretion signal (SS), FIVAR (found in various architectures) domain, two Siglec and unique domains, an LPxTG cell wall anchoring motif, and a series of DUF1542 (domain of unknown function) repeats (Fig. 1). The exact number of DUF1542 repeats in both *G. haemolysans* and *G. elegans* AsaA is unknown due to incomplete genomic sequences available on the NCBI. The NRR of AsaA begins after the SS and extends until the second unique domain ends, thus including the FIVAR domain and both Siglec and unique domains. Since the NRR of AsaA from IE12 acts as a sialic acid binding adhesin, the NRR from *G. haemolysans* and *G. elegans* AsaA may also act as an adhesin. Similarities in the amino acid content between the NRR from IE12 AsaA and the NRR of *G. haemolysans* and *G. elegans* AsaA also suggests similarities in the function of the protein. The percent amino acid identity between the NRR of IE12 AsaA and the NRR of AsaA in *G. haemolysans* M341 and *G. elegans* ATCC 700633 is 62% and 64%, respectfully. In addition, the NRR of *G. haemolysans* AsaA and the NRR of *G. elegans* AsaA share 77% amino acid identity.

A



B

	S. oralis	G. haemolysans	G. elegans
S. oralis	100%	62%	64%
G. haemolysans	62%	100%	77%
G. elegans	64%	77%	100%

Predicted aa identity of AsaA orthologs

Fig 1. AsaA orthologs exist in other IE causing species (A) Visual depicting the protein domains of AsaA: SS, secretion signal; FIVAR, found in various architectures; S, Siglec (sialic acid-binding immunoglobulin-type lectin); U, unique domain; LPxTG, cell wall anchoring motif; NRR, non-repeat region (B) Predicted amino acid identity for the NRR of AsaA across various IE causing species.

One strain of *G. elegans* and five strains of *G. haemolysans* are available on the NCBI. Of the five strains of *G. haemolysans*, only strain NCTC10459 is a complete genome. Only strain M341 encodes for AsaA however, due to the incomplete genomic sequences available, the presence of *asaA* within these genomes cannot be ruled out. Other strains of *G. haemolysans* may contain AsaA or an adhesin that promotes the development of IE if the conditions are correct. *G. haemolysans* AsaA was selected for further study. Directly upstream of *asaA* in *G. haemolysans* M341 is a gene predicted to encode for a nucleotidyl transferase and directly downstream is a gene with an unknown function. The genomic location of AsaA in *G. haemolysans* appears to be

different than AsaA in IE12 based on the identification of the genes flanking AsaA in both species. In both IE12 and *G. haemolysans,* the Siglec-like domains of AsaA are postulated to mediate binding to sialic acid. The Siglec-like and unique domains of IE12 AsaA shares a 72% amino acid identity with the Siglec-like and unique domains of *G. haemolysans* M341 AsaA (Fig. 2). This similarity suggests that like IE12 AsaA, *G. haemolysans* AsaA mediates adherence to sialic acid on platelets. If AsaA is a conserved mechanism of adhesion, a single treatment or preventative measure may target multiple IE causing species.

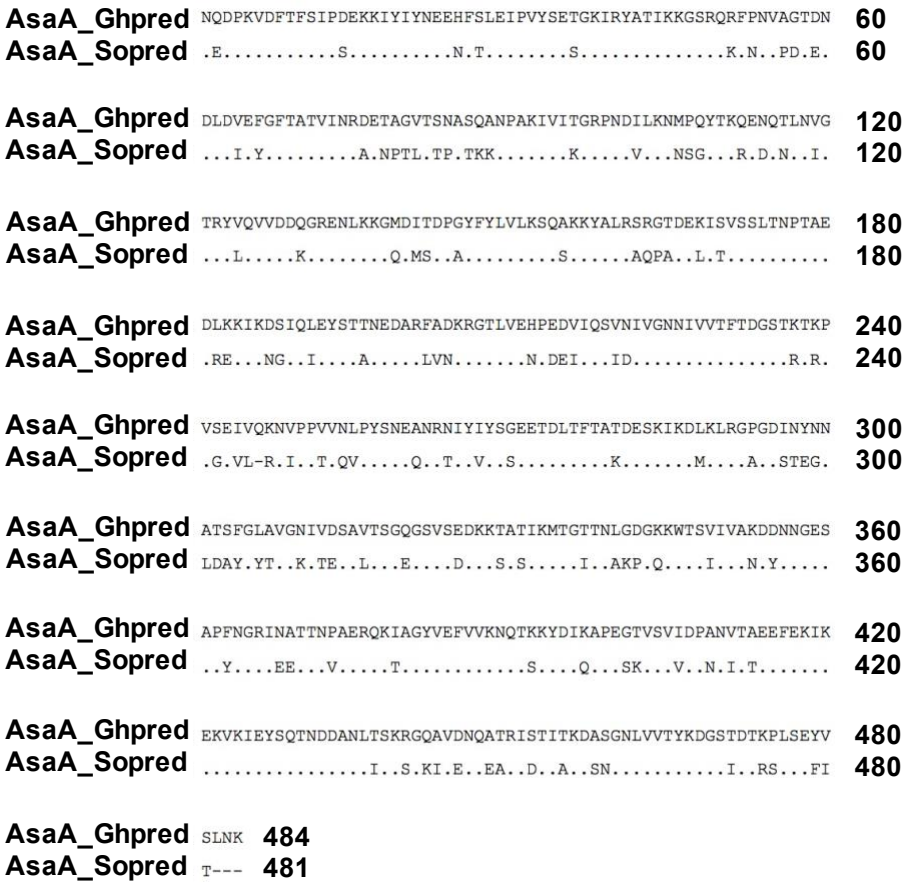


Fig 2. The Siglec-like and unique domains of IE12 AsaA share a high level of sequence conservation with the Siglec-like and unique domains of *G. haemolysans* AsaA. Amino acid alignment between the predicted Siglec-like and unique domains of AsaA in *G. haemolysans* and *S. oralis* IE12. The predicted amino acid location is given at the end of each line. A **.** indicates a conserved amino acid residue and a **-** indicates a gap in the sequence.

The addition of Fetal Bovine Serum (FBS) enhances the growth of *G. haemolysans in vitro*

Growth conditions for *G. haemolysans* needed to be optimized in order to observe significant adherence of the species to platelets, which would allow investigation of the role of sialic acid and AsaA in the adhesion of *G. haemolysans.* In order to maximize the adherence of the species to platelets, *G. haemolysans* was grown to various optical densities (OD_{600}) in BHI, the growth medium recommended by the Biodefense and Emerging Infections (BEI) resource repository. The absolute adherence, expressed as a percentage relative to the inoculum, of *G. haemolysans* to platelets was calculated at an OD_{600} of 0.4, 0.5, and 0.6. The inoculums were in the range of 2.33×10^5 to 3.33×10^5 bacteria. The absolute adherence of *G. haemolysans* to platelets at an OD_{600} of 0.4, 0.5, and 0.6 was 2.3%, 4.5%, and 1.9% respectfully. An OD_{600} of 0.5 resulted in the maximum observable adherence of *G. haemolysans* to platelets. However, in order for the species to reach this OD_{600} it had to be left to grow overnight, which prompted attempts to enhance the growth in vitro. A prior study had revealed that fetal bovine serum (FBS) increased the growth rate of *G. haemolysans.* (50). The growth of *G. haemolysans* was measured both with and without FBS. The addition of 5% FBS to growth media greatly enhanced growth (Fig. 3) and as a result was used for all future experiments.

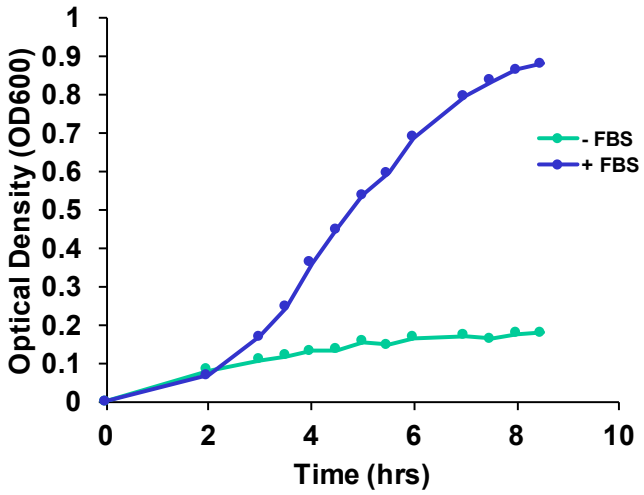


Fig 3. The addition of FBS enhances the growth of *G. haemolysans in vitro.* Growth curve for *G. haemolysans* with and without 5% FBS. Optical density was measured at a wavelength of 600 nm.

Attempts to construct an *ΔasaA* mutant in *G. haemolysans* using natural transformation were unsuccessful

In comparison to other IE causing bacterial species, *G. haemolysans* has rarely been studied. Previous literature on *G. haemolysans* is mainly limited to case reports and cases of IE caused by *G. haemolysans* are rare (39, 51, 52). Furthermore, there are no genetic tools available for *G. haemolysans*. Our attempts to naturally transform the species were unsuccessful which prevented the construction of an Δ*asaA* mutant. If significant binding of *G. haemolysans* to platelets was observed, an Δ*asaA* mutant would have permitted the direct assessment of the role of AsaA in adhesion of this species.

Low binding of *G. haemolysans* to platelets

To investigate the role of *G. haemolysans* AsaA in binding, the NRR of AsaA was recombinantly expressed (AsaA_NRGh). The initial strategy was to compete binding of *G.*

haemolysans to platelets with AsaA_NRGh. The ability of this protein to competitively inhibit the binding of *G. haemolysans* to platelets would support the hypothesis that *G. haemolysans* AsaA acts as an adhesin. However, low binding of *G. haemolysans* to platelets prevented testing the adherence of this species in the presence of AsaA_NRGh. The absolute adherence of *G. haemolysans* to platelets was on average 3.5%, whereas the absolute adherence for IE12 to platelets was around 11% (Fig. 4). The low binding of *G. haemolysans* to platelets and the variability in binding in the presence of neuraminidase (enzyme that cleaves sialic acid) prevented the assessment of the role of sialic acid in adhesion of *G. haemolysans.* The reason for low binding of *G. haemolysans* is unclear and one possible reason is that proteins such as AsaA are not being expressed under our *in vitro* conditions.

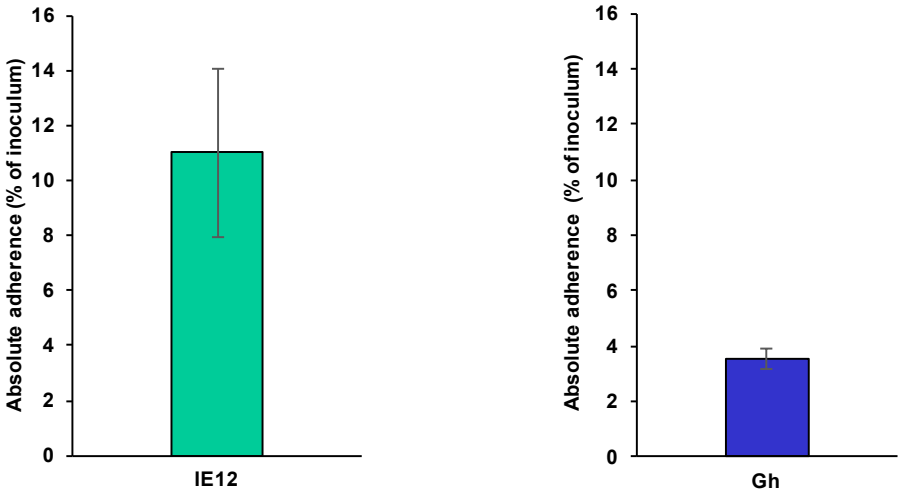


Fig 4. Absolute adherence of *S. oralis* IE12 and *G. haemolysans* M341. Absolute adherence, expressed as a percentage of the inoculum, of bacterial species to platelets. Values are the means of at least three independent experiments performed in triplicate +/- SD.

Recombinantly expressed AsaA_NRGh competitively inhibits binding of *S. oralis*

Prior experimentation demonstrated AsaA_NRSo competitively inhibited binding of IE12 to platelets. These experiments were adapted in order to determine if AsaA_NRGh could also block

adhesion of IE12, which would support the hypothesis that *G. haemolysans* AsaA acts as an adhesin. Relative adherence, expressed as a percentage, of the IE12 parental strain was calculated for each condition. As expected, the positive control, 5 μM AsaA_NRSo, competitively inhibited adherence of IE12 to platelets (Fig. 5). AsaA_NRGh (5 μM) also significantly reduced adhesion of IE12 to platelets (Fig 5). AsaA_NRSo and AsaA_NRGh reduced the adherence of IE12 to a similar extent which supports the hypothesis that these two species share a conserved mechanism of adhesion.

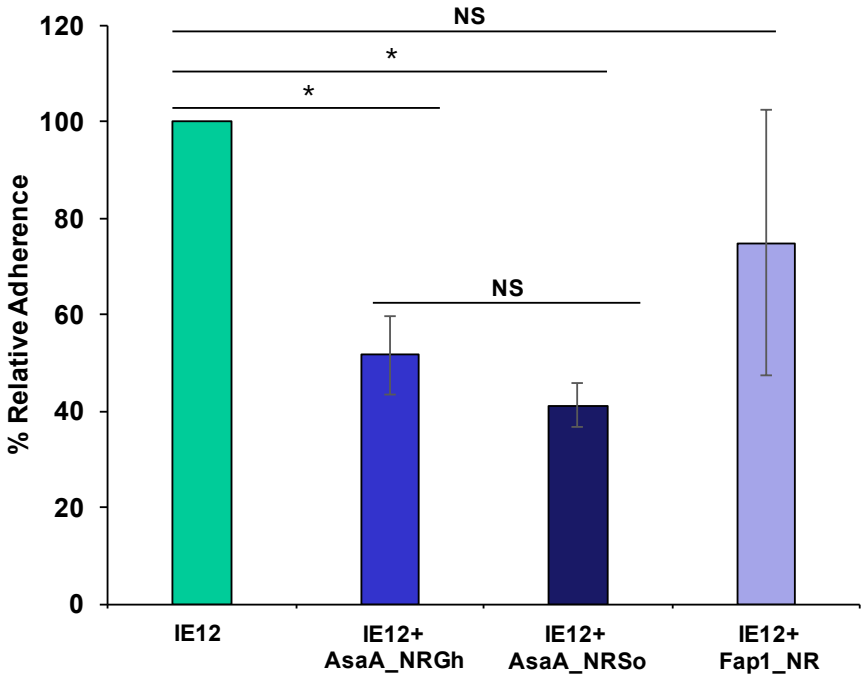


Fig 5. Recombinantly expressed AsaA_NRGh significantly reduces binding of IE12 to platelets. Relative adherence, expressed as a percentage of IE12 binding in the presence and absence of recombinant protein. Values are the means of at least three independent experiments performed in triplicate +/- SD. *p<0.05, calculated using a two tailed t-test. NS, not significant

To examine if Fap1 and AsaA share a conserved mechanism of adhesion, adherence of IE12 was tested in the presence of the recombinantly expressed NRR from Fap1 (Fap1_NR). Fap1 is an SRRP that mediates adherence to sialic acid through its single Sigec-like domain. Fap1_NR

competitively inhibits adhesion of Fap1 encoding strains (30). However, there was no significant reduction in adhesion of IE12 in the presence of Fap1_NR which could be due to the low level of sequence identity between Fap1 and AsaA in both *S. oralis* and *G. haemolysans* (Fig. 6).

Fig 6. Low level of sequence identity between the first Siglec-like and unique domain of IE12 and *G. haemolysans* AsaA and the Siglec-like and unique domain of Fap1. Amino acid alignment between the first predicted Siglec-like and unique domain for AsaA in *G. haemolysans* and *S. oralis* IE12 and the predicted Siglec-like and unique domain for Fap1. A **.** indicates a conserved amino acid residue and a **-** indicates a gap in the sequence.

The Siglec-like and unique domain of Fap1 shares higher levels of sequence similarity with the first Siglec-like and unique domains than the second Siglec-like and unique domains from *S. oralis* and *G. haemolysans* AsaA. The Siglec-like and unique domain of Fap1 and the first Siglec-like and unique domain of AsaA in *G. haemolysans* and *S. oralis* IE12 only share 16%

amino acid identity (Fig. 6). The inability of Fap1 to significantly reduce binding of IE12 to platelets suggests a difference in sialic acid binding specificities of Fap1 and AsaA.

The reduction in adhesion of IE12 in the presence of AsaA_NRGh is AsaA dependent

The ability of AsaA_NRGh to competitively inhibit binding of *S. oralis* to platelets led to the question of whether this reduction was AsaA dependent. Once again, AsaA_NRGh (5 μm) competitively inhibited binding of IE12 to platelets. The Δ*asaA* IE12 mutant was utilized as a positive control and confirmed AsaA is required for efficient adhesion to platelets (Fig. 7). AsaA_NRGh (5 μm) did not significantly reduce adhesion of the mutant strain which demonstrates AsaA_NRGh competitively inhibits the binding of IE12 to platelets in an AsaA dependent manner (Fig 7).

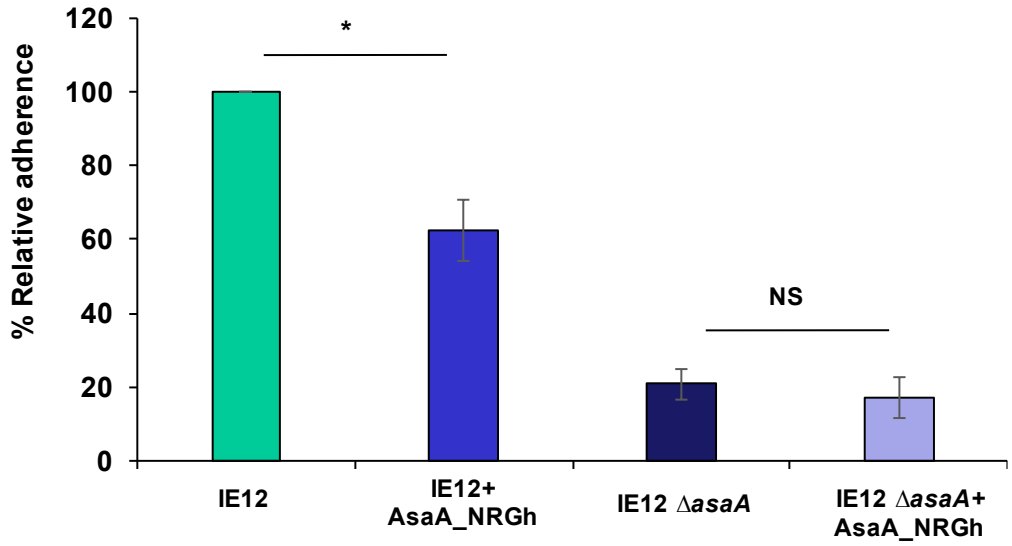


Fig 7. Recombinantly expressed AsaA_NRGh significantly reduces binding of IE12 to platelets in an AsaA dependent manner. Relative adherence, expressed as a percentage of IE12 binding in the presence and absence of recombinant protein. Relative adherence of an Δ*asaA* mutant in the presence and absence of recombinant protein is also shown. Values are the means of at least three independent experiments performed in triplicate +/- SD. *p<0.05, calculated using a two tailed t-test. NS, not significant

DISCUSSION

The pathogenesis of subacute IE is not completely understood. Although understudied, a critical step in the development of IE is bacterial adherence to platelets. Multiple IE causing species encode for an SRRP and/or AsaA, both of which are sialic acid binding adhesins. These findings demonstrate that blocking the binding of IE causing bacterial species to sialic acid may be a potential drug target. In this study, we examined if AsaA orthologs exist in other IE causing species and mediated adhesion to platelets. AsaA orthologs were identified in IE causing species *G. haemolysans* and *G. elegans.* The recombinantly expressed NRR of AsaA from *G. haemolysans* competitively inhibits binding of IE12 to platelets in an AsaA dependent manner. This finding demonstrates a potential conserved mechanism of adhesion between *G. haemolysans* AsaA and IE12 AsaA.

Previous studies have demonstrated the importance of SRRPs in adherence of IE causing bacterial species to sialic acid. Some strains of viridans group streptococcal species *S. gordonii*, *S. sanguinis*, and *S. oralis* bind to sialic acid via SRRPs that contain a Siglec-like domain (13, 18, 20-22). *S. oralis* IE isolates IE1, IE12, and IE18 bind to sialic acid, but lack Fap1. Genomic sequencing of IE12 and comparative genomics revealed a protein, AsaA, predicted to contain Siglec-like domains. Given the importance of these domains in mediating adherence to sialic acid in IE causing species, the role of AsaA in adhesion was examined. AsaA in IE12 was demonstrated to act as a sialic binding adhesin. An NCBI BLASTp using the amino acid sequence for the Siglec-like and unique domains in IE12 AsaA revealed similar sequences in *G. haemolysans* M341 and *G. elegans* ATCC 700633 (49). A review of the literature about these species revealed both *G. haemolysans* and *G. elegans* are causative agents of IE (40, 44-46, 53). ATCC 700633 is the only *G. elegans* strain sequenced on the NCBI, but there are four additional

strains other than *G. haemolysans* M341 sequenced. The sequences of these strains are incomplete and hence they may encode for AsaA. Alternatively, they may encode for a different adhesin that promotes the development of IE if the conditions are correct or it is possible only some strains of this species have the ability to cause IE. IE12 AsaA, *G. haemolysans* AsaA, and *G. elegans* AsaA all contain Siglec-like and unique domains of similar amino acid lengths and sequences which lead to the hypothesis that AsaA is a family of sialic acid binding adhesins present in various IE causing species. A previous study demonstrated both the Siglec and unique domains of an SRRP were required for sufficient sialoglycan binding in *S. gordonii* (18). Thus, the unique domains in AsaA may play a role in mediating adherence to platelets which prompts future attempts to isolate their function. DUF 1542 domains have been found in cell surface proteins that play a role in antibiotic resistance or cellular adhesion (54). Specifically, these domains have been found in *S. aureus* EbhA, a protein that binds to human fibronectin (55). In addition, a previous study demonstrates DUF1542 repeats have been found in Epf (Extracellular protein factor) in *Streptococcus pyogenes,* another IE causing species. These repeats form a fiber-like stalk which projects the adhesin domain towards host receptors. Thus, DUF1542 repeats found in AsaA may assist in projecting the Siglec-like and unique domain towards host surfaces (56).

G. haemolysans AsaA was selected for further study. Examination of the genes that flank *asaA* in *G. haemolysans* revealed differences in the genomic location of *asaA* between this species and *S. oralis* IE12. The gene directly upstream of *asaA* encodes for a nucleotidyl transferase in *G. haemolysans* and for a peptidase in IE12. The gene directly downstream encodes for an amino transferase in IE12, while incomplete genomic sequences available for *G. haemolysans* M341 prevent identification of the function of the gene directly downstream.

In order to investigate the role of *G. haemolysans* AsaA in adhesion, growth conditions of the species were optimized. Brain Heart Infusion (BHI) Broth was suggested as the growth conditions for *G. haemolysans* M341 by the BEI. BHI alone did not support efficient growth of *G. haemolysans.* Investigation of previous attempts to grow *G. haemolysans in vitro* revealed FBS was used to enhance the growth (50). FBS is widely used as a growth supplement for cell culture media and provides many components that satisfy the metabolic requirements for cells (Sigma Aldrich) Although FBS is primarily used in eukaryotic cell cultures, it has been used in liquid cultures of bacterium such as *Helicobacter pylori* (57). Thus, the previous use of FBS to enhance the growth of *G. haemolysans* was not unexpected. The addition of 5% FBS greatly enhanced both the growth rate and growth yield of *G. haemolysans.* However, the addition of the serum may result in unintended effects. FBS could potentially affect the gene expression of *G. haemolysans* and causes changes in the expression of genes including AsaA. Previous literature has demonstrated serum influences exopolysaccharide expression in *Staphylococcus aureus,* a leading cause of acute IE (58). An additional study reveals virulence factors in *S. aureus* were significantly expressed in the presence of serum (59). The effect of FBS on gene expression in *G. haemolysans* remains unknown.

The observed low binding of *G. haemolysans* to platelets could be explained by changes in expression of genes such as *asaA* under our *in vitro* conditions. The gene expression of *G. haemolysans* under our *in vitro* conditions may not reflect that *in vivo*. Future experiments should be aimed at optimizing the binding of the species to platelets. Inability of the species to bind to platelets at a significant level prevents the use of mutants, recombinant proteins and enzymatic treatments to investigate the adhesion of WT *G. haemolysans*. Optimizing the binding of *G. haemolysans* would permit these experiments and the assessment of the role of AsaA in *G.*

haemolysans. We were unable to generate an *asaA* mutant through natural transformation. Although attempts to naturally transform the species were unsuccessful, the species may still be capable of transformation despite the lack genetic tools available for *G. haemolysans*. In future experimentation, alternative methods such as electroporation could be explored to generate mutants.

Our studies demonstrate AsaA_NRGh can competitively inhibit binding of IE12 to platelets. AsaA_NRGh and AsaA_NRSo reduced the adherence of IE12 to platelets to a similar extent, which may be a reflection of the high level of sequence conservation between the two proteins. This finding suggests a conserved mechanism of adhesion for AsaA in *G. haemolysans* and *S. oralis* IE12. However, Fap1_NR did not significantly reduce the adhesion of IE12 to platelets. Fap1 only contains one Siglec, while AsaA contains two (30). The amino acid identity between the Siglec in Fap1 and the first Siglec in *G. haemolysans* AsaA and IE12 AsaA reveals low levels of sequence conservation (Fig. 6) Thus, Fap1 and AsaA may have different sialic acid binding specificities. Sialic acids are a diverse group of sugars. The nine carbon backbone is common to all sialic acids, but there are variations that can occur at different carbon positions (60). Substitutions occur at the 4, 5, 7, 8, and 9 positions and variations in linkages to the underlying sugar chain occur at position 2 (61,62). Thus, Fap1 could bind to a different sialic acid or sialic acid linkage than AsaA. If this is true, blocking IE12 binding with Fap1 would be ineffective as IE12 AsaA would bind to a different sialic acid or sialic acid linkage than Fap1.

There was no significant difference in the adhesion of the IE12 Δ*asaA* mutant with and without the presence of AsaA_NRGh. Thus, AsaA_NRGh competitively inhibits adhesion of IE12 to platelets in an AsaA dependent manner. This finding demonstrates the importance of AsaA as a

mechanism for binding to sialic acid and suggest similarities in the binding specificities of IE12 AsaA and *G. haemolysans* AsaA.

Our data suggests we have identified a novel family of sialic acid binding adhesins present in various IE causing species. Future efforts could be focused on optimizing the binding of *G. haemolysans* to platelets in order to test adherence with the enzymatic removal of sialic acid. A significant reduction in adherence would demonstrate sialic acid is required for maximum binding of *G. haemolysans* to platelets, supporting the importance of sialic acid binding adhesins in the pathogenesis of IE. In addition, the binding region of AsaA from IE12 could be replaced with the binding region of AsaA in *G. haemolysans* to see if this domain also mediates adherence. The role of *G. elegans* AsaA in adhesion could also be examined in order to increase our understanding of the role of AsaA in adhesion. Continuation of the work to examine if AsaA is a conserved mechanism of adhesion across IE causing species could reveal broader clinical significance and improve understanding of the pathogenic mechanisms utilized by IE causing species.

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